Incubation temperature affects type X collagen expression in embryonic ducks

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May 2014

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Abstract

Optimal temperature regulation is an important component of avian incubation and the process of embryonic development can be accelerated by even small increases in incubation temperature. The incubation period for ducks is 28 days and it has been shown in our lab that an increase in temperature from 37.5 °C to 38.5 °C during early incubation (0 to 10d) will increase embryonic body weight during mid-incubation (approximately 10 to 18d) in duck embryos. The aim of this project was to study the effects of increased incubation temperature on type X collagen expression in duck embryos. Type X collagen is a marker specific to hypertrophic chondrocytes which are associated with the terminal maturation of cartilage prior to the onset of mineralization. The femur and tibiotarsus of White Pekin ducks were collected at various stages of embryonic development. RNA was isolated from these tissues and used to make corresponding complementary DNA. This cDNA was then used for a polymerase chain reaction (PCR) assay with a primer for type X collagen found in the literature. The primers for TBP from *Meleagris gallopavo* and RPS13 for *Anas platyrhynchos* were used as housekeeping genes. It was observed that type X collagen was expressed in embryos collected from both temperature treatments as early as day 12 of incubation. The preliminary data resulting from image analysis suggests that an increase in incubation temperature increases the transcription of type X collagen in mid to late embryogenesis, 18d and 25d. However, at hatch, transcription of type X collagen in birds incubated at 99.5 °F is greater than those incubated at 100.5 °F. These results suggest a potential positive relationship between increased embryonic transcription of type X collagen and embryo growth.

Introduction

Decuypere and Michels (1992) reported that within the poultry industry, the increasing size of commercial incubators can result in variability in the temperature experienced by growing embryos. Embryos in one section of an incubator may be exposed to periods of higher temperature than those in a different section. The heat within an incubator is due to both embryonic metabolic heat production as well as exogenous heat from the incubator itself (Lourens et al., 2011). The effect of incubation temperature on embryonic development and hatchability in poultry species was initially reported over 70 years ago and it was concluded that the optimum temperature for chicks is 37.5°C (99.5 °F; Romanoff, 1937). Romanoff determined that there was a progressive decline in hatchability when embryos were exposed to temperatures above 38˚C and below 37˚C for extended periods during the incubation process.

It was subsequently speculated that the relationship between incubation temperature and its effect on embryonic development may play a key role in many chick quality problems encountered today. French (1997) reported that during mid-development, embryo temperature rises above incubation temperature due to metabolic heat production. Bowers and Lilburn (2006) reported that temporary increases in incubation temperature to 38.5°C from an optimal level of 37.5°C during the initial stages of embryonic development accelerated embryonic growth rate and skeletal development. These short term increases in incubation temperature increased dry embryo weights through 12 days of incubation in White Pekin Ducks. Any deviation from optimal incubation conditions may result in negative post-hatch effects on

thermoregulatory control and skeletal development in chicks (Bowers and Lilburn, 2006).

Cartilage, the precursor to most bones, is derived from the mesoderm during avian embryogenesis. This cartilage is eventually replaced by bone through a process called endochondral ossification. Chondrocytes become active and swell to become hypertrophic chondrocytes during the endochondral ossification process. Type X collagen is a marker protein that is produced by hypertrophic chondrocytes (Linsenmayer and Schmid, 1985). The production of type X collagen increases progressively during the course of embryonic skeletal development and thus type X collagen was hypothesized to be a suitable marker for this study.

Decuypere and Michels (1992) reported that embryonic development occurs at disproportionate rates at incubation temperatures lower than optimum (from 27°C to 35°C). This could be due to differing threshold temperatures for different tissues. Temperatures below the “physiological zero” (27°C) could be used for longer-term storage because development as a whole is slowed nearly to a halt (Decuypere and Michels, 1992). While this work considered the effects of lowering the incubation temperature during incubation, there is no hypothesis describing the parallel physiological effects of short periods of elevated incubation temperatures.

The increase in dry embryo weights in duck embryos incubated at 38.5 °C versus 37.5 °C from 0 to 10d disappeared by approximately day 22 of incubation (Bowers and Lilburn, 2006). It is unknown whether this short acceleration of early growth causes permanent changes in skeletal development or if the skeletal system returns to a steady

state by the latter stages of incubation. At the older embryonic ages, the shell may also become a physical barrier to continued acceleration in embryonic growth. Bowers and Lilburn (2006) concluded that embryos exposed to the lower control temperature (37.5°C) during the entire incubation period complete the incubation with similar weights to those embryos exposed to the higher temperature. However, there have been no studies investigating the mechanisms of incubation temperature on bone development.

Based on the preliminary work by Bowers and Lilburn (2006), the ultimate aim of this project was to determine the effects of increasing incubation temperature to 100.5 °F from 99.5 °F on type X collagen expression in embryonic ducks. Housekeeping genes were first identified based on degenerate sequence analysis in order to semi-quantify type X collagen expression. It was hypothesized that the expression of type X collagen would be accelerated in those embryos incubated at a temperature of 100.5 °F versus those incubated at 99.5 °F.

Methods

Bone Samples

Duck eggs were marked and placed in two incubators set at either 100.5°F or 99.5°F. At the time of sample collection, eggs were weighed, followed by the extraction and euthanasia of the embryos, and then the recording of embryonic yolk-free body weight. Samples of tibiotarsus and femur were harvested from White Pekin duck embryos at days 4, 5, 6, 7, 8, 12, 18, 25 of incubation and 1 day posthatch. At embryonic days (ED) 4, 5,6,7,8, and 12, the size of the embryos did not allow for the dissection and extraction of individual bones, and so the entire lower half of the embryo was placed in liquid nitrogen for further RNA isolation. Embryos at day 18, 25, and 1

day posthatch were large enough that the tibiotarsus and femur could be collected and placed in liquid nitrogen for subsequent RNA isolation. All samples were stored long-term at -80 °C.

The RNA was extracted using an Animal Tissue Purification Kit by Norgen Biotek Corporation and stored at -80°C. The integrity of the samples of RNA was tested by running them on 1.5% Tris/Acetic acid/EDTA (TAE) agarose electrophoresis gel. Gel images were photographed under ultraviolet light to observe bands representing the 18S and 28S subunits of ribosomal RNA molecule.

PCR

The RNA samples were subsequently used to create corresponding single stranded complementary DNA (cDNA) using an anchored oligo dT primer [oligo (dTA)]. This template cDNA was used in PCR with primers for type X collagen designed based on the literature (Chang, Cheng, and Huan, 2012). In two additional, separate reactions, the same template cDNA was mixed with degenerate primers for a TATA Box Binding Protein (TBP) gene designed from the predicted sequence in *Meleagris gallopavo* and primers for Ribosomal Protein S13 (RPS13) in *Anas platyrhynchos*. PCR consisted of a series of 34 cycles of denaturation at 95 °C, primer annealing at 59 °C, and template extension at 72 °C. The primer sequences used were:

Primer Name	Forward Primer Sequence[1]	Reverse Primer Sequence[1]	Accession Number	Product Size	Species
COLX	CTGGCAGTGC TGTCATCGAT	GCGTGACCTCCTA AAGGACATC	XM_005025230	220 bp	*Anas platyrhynchos*
TBP	TGTCCAGAGCA CCAACAGTC	AGGGGTACAGAGG TGTGGTT	XM_003204046	188 bp	*Meleagris gallopavo*
RPS13	CAAGAAGGCT GTTGCTGTTCG	GGCAGAAGCTGTC GATGATT	XM_005015078.1	169 bp	*Anas platyrhynchos*

Table 1: PCR primer sequences and expected product sizes; [1]sequences listed 5’ to 3’

The identification of the two housekeeping genes ultimately used for analysis resulted from several PCR experiments conducted in a similar manner as previously described. Table 2 summarizes the process.

	4d		11d	12d	
	99.5 °F	100.5 °F	99.5 °F + 100.5 °F	99.5 °F	100.5 °F
HMBS	(-) Smear	(+) Multiple Bands	(+) Multiple Bands	--	--
HPRT	(-)	(-)	(+)	--	--
RPL4	(-) Smear	(-)-Smear	(+)	--	--
GAPDH	Inconsistent	Inconsistent	(+)	--	--
TBP	(+)	(+)	--	(+)	(+)
RPS13	(+)	(+)	--	(+)	(+)

Table 2: Housekeeping gene experimentation. 4d embryos are representative of early embryonic stages. 11d and 12d embryos are representative of mid to late embryogenesis. (+) indicates consistent expression of the particular gene, while (-) indicates inconsistent expression.

Electrophoresis

PCR products were analyzed using electrophoresis by mixing 5 µL of sample and 2 µL of loading dye and run on 1.5% (TAE) agarose electrophoresis gel. As a reference for amplicon size, a 100 bp ladder was run alongside the samples. Table 1 shows the fragments produced by each primer. Ultraviolet light was used to examine and photograph the gels.

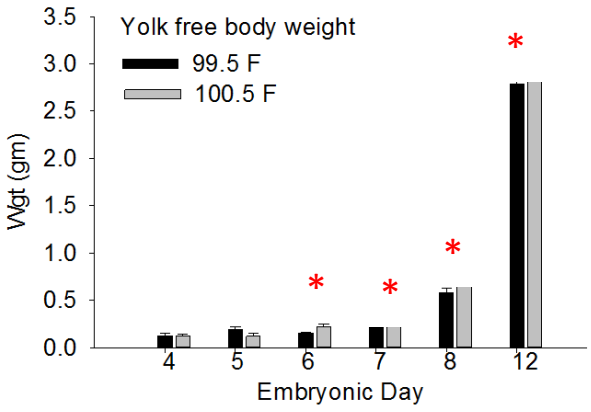
Image Analysis

Gel images were digitized and subsequently analyzed using TotalLab Quant Software in order to semi-quantify type X collagen gene expression relative to the RPS13 housekeeping gene. A PCR experiment identical to that shown in Figures 3-5

(Results section) was performed in triplicate for statistical analysis. Using the software, the volume of each band on the photograph was determined. The volume of each type X collagen band was expressed as a percentage of each of the RPS13 bands, for a total of nine values for each embryonic age and treatment temperature. A t-test was then conducted to identify any difference between incubation temperature treatments for each sampling age (Table 3).

Results

Significant differences in embryonic yolk-free body weight were observed beginning at 6d of age (Figures 1 and 2). Those birds incubated at 100.5 °F were significantly heavier than those birds incubated at 99.5 °F at days 6, 7, 8, and 12. These differences disappeared at days 18 and 25 but, were observed again at hatch (Figures 1 and 2).



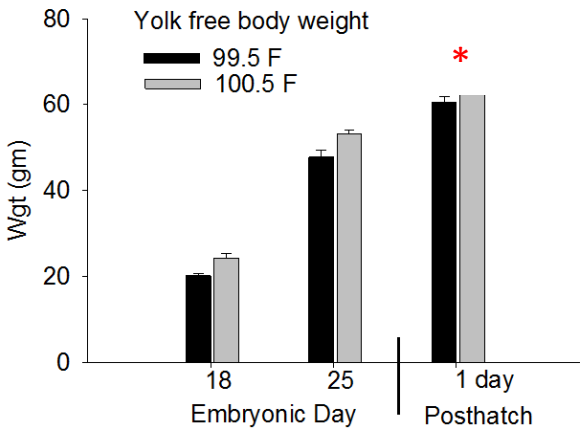


Figure 1: Yolk-free body weight, 4-8d, 12d,
Figure 2: Yolk-free body weight, 18d, 25d, 1d Posthatch.
* denotes p<0.05, showing significant differences in body weight.

Type X collagen expression was first observed at day 12 of incubation, and was expressed in both incubation temperature treatment groups. The expression of type X collagen in embryos 4d-12d, accompanied by TBP and RPS13 housekeeping genes is shown in Figures 3 and 4. The data in Figure 4 shows that type X collagen expression begins in 12d embryos in both treatment groups and is present in every treatment group thereafter, as seen in Figure 5.

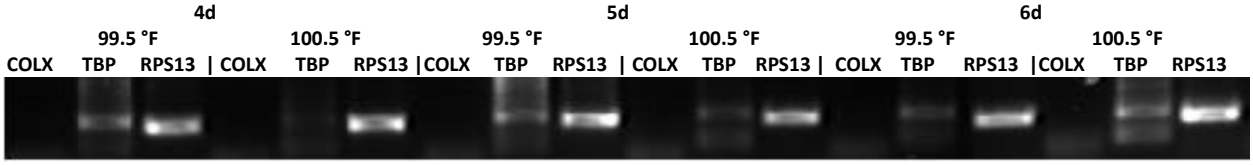


Figure 3: Expression of type X collagen and housekeeping genes, TBP and RPS13 in ED 4-6.

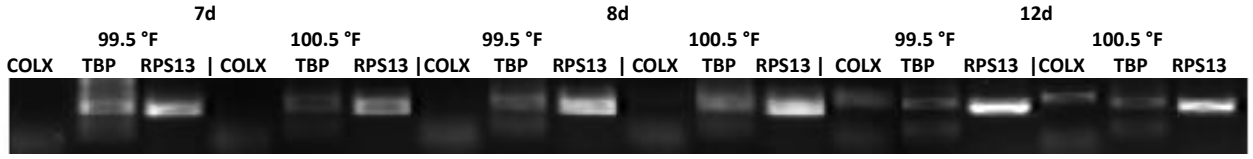


Figure 4: Expression of type X collagen and housekeeping genes, TBP and RPS13 in ED 7-12.

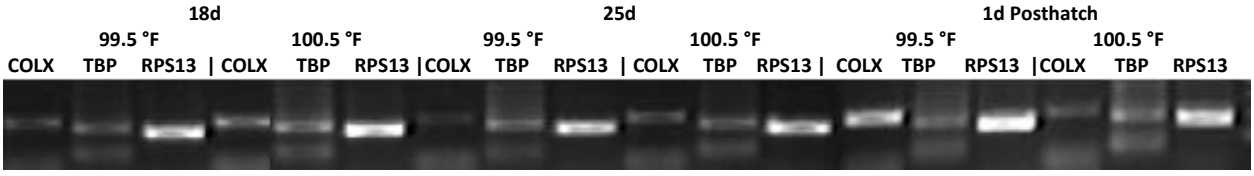


Figure 5: Expression of type X collagen and housekeeping genes, TBP and RPS13 in ED 18 & 25 & 1d posthatch.

This PCR experiment was subsequently repeated in triplicate for appropriate statistical analysis and the results are shown in Table 3.

Day	Treatment	Relative COLX Expression	*P*-value
12	99.5 °F	24.58%	*P*= 0.72
	100.5 °F	22.03%	
18	99.5 °F	32.60%	*P*<0.0001
	100.5 °F	89.47%	
25	99.5 °F	44.36%	*P*<0.0001
	100.5 °F	70.97%	
1d Post	99.5 °F	97.29%	*P*<0.0001
	100.5 °F	59.73%	

Table 3: Relative quantitative expression of type X collagen, expressed as a percentage of RPS13 expression.

Discussion

In addition to the objective of observing the effects of incubation temperature on type X collagen expression, a secondary but equally important objective of this study was to identify suitable housekeeping genes for use in semi-quantifying type X collagen expression. This objective proved to be more difficult than initially planned. When designing primers for housekeeping genes, one must use either the predicted sequence for the test species, in this case ducks, or go to another avian species. This process can be time consuming and often involves a great deal of trial-and-error.

Several housekeeping genes were used in a series of experiments, including hydroxymethylbilane synthase (HMBS), Hypoxanthine-guanine phosphoribosyltransferase (HPRT), Ribosomal Protein L4 (RPL4), and Glyceraldehyde

3-phosphate dehydrogenase (GAPDH) from *Gallus gallus,* as described in Table 2*.* Housekeeping genes are generally expressed in all cells of a particular tissue or species under normal conditions. In addition, these genes should be expressed consistently to allow for quantitative comparisons to be made between the housekeeping genes and the gene of interest over time. In the case of GAPDH, it is possible that in early embryogenesis several isoforms of the gene may exist. More work is warranted to investigate these possibilities. As seen in Figures 3-5 above, TBP and RPS13 were expressed ubiquitously across all embryonic stages as well as posthatch, thus making them suitable housekeeping genes for experiments involving duck embryogenesis.

It should also be noted that two procedures were utilized to isolate RNA at different points during the completion of this project. The first, using Trizol® reagent and the associated protocols produced RNA that we suspected to have been contaminated with DNA. The RNA and subsequent cDNA produced from these procedures was used in PCR reactions using the same COLX primers identified in the experiments previously mentioned. However, the templates produced from the Trizol® procedure showed type X collagen expression at every stage of embryogenesis, as early as 4d in both temperature treatments. The subsequent attempt to verify these results used RNA isolated using the Norgen Animal Tissue RNA Purification Kit. Amplicons from this source produced type X collagen expression first at 12d, results that are more consistent with those reported in the literature (Chang et al., 2012). The Norgen Kit protocol includes a step in which the RNA is washed with DNase, eliminating the problems associated with DNA contamination using the Trizol® protocol.

The onset of measurable type X collagen expression occurred at 12d in both treatments. Further analysis of the images produced from the triplicate reactions of the same PCR experiment (Figures 3-5) suggest that increases in incubation temperature increased the transcription of type X collagen at 18d and 25d as well (Table 3). At hatch, however, this was reversed; type X collagen expression in birds incubated at 99.5 °F was increased when compared with those ducklings incubated at 100.5 °F.

The trends in accelerated yolk-free body weight gain observed in previous studies in our lab (Bowers and Lilburn, 2006) were also observed in the current study (Figures 1 and 2). Ducks incubated at 100.5 °F had accelerated body weight gain compared to those ducks incubated at 99.5 °F at 6d, 7d, 8d, 12d, and hatch. When the yolk-free embryonic body weight data was combined with the relative type X collagen expression data, it can be concluded that there is a potential positive relationship between increased incubation temperature, accelerated yolk-free body weight gain, and increased transcription of type X collagen. It is possible that during mid to late incubation (12d to posthatch), increased transcription of type X collagen precedes accelerated body weight gain. Of course, this hypothesis is only relevant after the onset of type X collagen expression, which in the current experiment was day 12 of incubation.

In order to do a more complete statistical analysis of the relationship between incubation temperature, type x collagen transcription, and embryonic body weight gain, RNA must be isolated from more ducks. The results reported here were generated from the isolation of RNA from one bird per sample from embryonic ages 12d to hatch, and 2 birds per sample in ages 4-8d. Ideally, five birds per treatment per sample would be

needed to verify our results. The findings offered here can be used as a basis from which to move forward with similar work in order to paint a clearer picture of these physiological processes occurring during incubation.

Conclusions

This project identified two housekeeping genes that can be used for future research in duck embryogenesis, especially those studies focusing on skeletal development. Housekeeping genes specific to duck skeletal development in embryogenesis have not been reported prior to this project.

The results suggest a relationship between increased embryonic transcription of type X collagen and increased embryo growth, potentially that greater type X collagen expression in mid incubation precedes accelerated growth.

Our understanding of how incubation temperature affects skeletal development and overall growth can improve the manner in which we incubate avian species on a commercial level. These improvements could potentially be extrapolated to other species, as well. The determination of a relationship between incubation temperature, weight gain, and skeletal development, in addition to the identification of suitable housekeeping genes are important steps in the process of understanding ways in which we can improve upon our current practices.

Literature Cited

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